Intraspecific Differentiation of *Vibrio vulnificus* Biotypes by Amplified Fragment Length Polymorphism and Ribotyping

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The intraspecific genomic relatedness of 80 *Vibrio vulnificus* isolates, 44 of biotype 1 and 36 of biotype 2, from different geographic origins and sources was evaluated by ribotyping and AFLP (amplified fragment length polymorphism) fingerprinting. Ribopatterns of DNAs digested with *Kpn*I and hybridized with an oligonucleotide complementary to a highly conserved sequence in the 23S rRNA gene revealed up to 19 ribotypes in the species, which were different for the two biotypes. Sixteen different ribotypes were found within biotype 1 strains from clinical and environmental sources, and only three, recovered mainly from diseased eels, were found within biotype 2. Within this biotype, 96% of the strains showed the same ribopattern. The closest similarity was shown by the strains coming from the same eel farm, irrespectively of biotype. AFLP fingerprints obtained by selective PCR amplification of *Hind*III-*Taq*I double-restricted DNA fragments exhibited a strain-specific pattern which allowed the finest differentiation of subgroups within the eel-pathogenic isolates sharing the same ribopattern. Both techniques revealed good genetic markers for intraspecific differentiation of *V. vulnificus*. Ribotyping clearly separated the eel-pathogenic strains from the clinical and environmental isolates, whereas AFLP enabled the monitoring of individual strains and therefore constitutes one of the most discriminative tools for epidemiological and ecological studies.

Intraspecific characterization of pathogenic bacteria for epidemiological purposes can be achieved by several procedures. Phenotypic characterization, widely used in the past, takes into account only few characters and has proven to be nonreliable. In the last 2 decades, the development of molecular techniques that allow the comparison of strains by directly looking at their genomes has overcome the shortcomings of analyzing phenotypic properties. In this paper, we will deal with *Vibrio vulnificus*, an autochthonous marine and estuarine bacterium described as one of the most invasive and fulminantly lethal human opportunistic bacterial pathogens (13, 21, 24–26). The species also comprises fish-pathogenic strains, originally isolated from diseased Japanese eels, that are responsible for severe epizootics at eel farms.

The first attempt at differentiation within the species *V. vulnificus* was made by Tison et al. (34), who defined two biotypes or biogroups to separate the strains pathogenic for eels (biotype 2) from those pathogenic for humans (biotype 1). Biotype 2 strains were serologically homogeneous, and apart from their host range, they could be distinguished by several phenotypic features (34). Since the description of biotype 2, and except for a few reports of Japanese eel isolates (16, 34), studies of *V. vulnificus* have been done with clinical or environmental isolates recovered from the western and eastern U.S. coasts (21–25). It was not until 1991, when Biosca et al. (8) reported the isolation of biotype 2 from diseased eels in a European farm and carried out several studies of its virulence factors (1–3, 10, 14). In those studies, they found that both biotypes share the main virulence factors already described for biotype 1 but that biotype 2 is pathogenic for mice as well as for eels, a character not shown by biotype 1 strains (3, 9). It was

also found that the absence of indole production was the only phenotypic feature of those defined by Tison et al. that remained reliable for biotype 2 (9). Recently, Amaro and Biosca have ascribed to biotype 2 a clinical strain (ATCC 33817) originally isolated from a wound infection (5). In that report, the authors stressed the high phenotypic similarity between the two biotypes and suggested that it could be the reason for misidentifications. In fact, an indole-negative strain has been associated with human infections after handling of diseased eels (36). Although biotype 2 strains have always been recovered from diseased eels and seem to be obligate fish pathogens, evidence of its survival in brackish water under laboratory conditions has been reported recently (4).

The proper separation of eel- and human-pathogenic strains, as well as the possibility of tracking specific strains, is essential to an understanding of the ecology and transmission route of human- and fish-pathogenic strains. DNA fingerprinting techniques like pulsed-field gel electrophoresis (PFGE), ribotyping, random amplification of polymorphic DNA, or amplified fragment length polymorphism (AFLP), whose high discriminative capacity allows investigators to distinguish among isolates, are currently being used for such purposes (11, 12, 15–19, 27, 28, 31, 33). Of the different techniques available, we have chosen ribotyping, which has been widely used in studies of human and fish pathogens (12, 15, 27, 28, 31, 33), to compare with the recently described AFLP fingerprinting technique (17–20, 37). Ribotyping is a powerful and reliable procedure which consists of the selective hybridization of ribosomal DNA (rDNA) genes on total DNA restricted fragments separated by electrophoresis and blotted onto a membrane. It allows easy reading and is based on stable genetic characteristics (rRNA genes present in all bacteria). AFLP is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: (i) restriction of the DNA and ligation of oligonucleotide adapters, (ii) selective amplification of sets of restriction frag-

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ments, and (iii) gel analysis of the amplified fragments. It is one of the most sensitive discriminatory techniques available for assessing intraspecific variability and has been used in a variety of species that include environmental and clinical strains (17, 18, 20).

In the present work, we have used both techniques for intraspecific differentiation of 80 strains of *V. vulnificus* originating from different geographic areas and belonging to biotypes 1 and 2 (i) to compare their discriminative powers, (ii) to search for reliable markers within the species, and (iii) to study intraspecific genetic variability.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Eighty strains of *V. vulnificus* biotypes 1 and 2, including reference strains, were used (Table 1). Strains were grown in Nutrient Agar (Oxoid) supplemented with 0.5% (wt/vol) NaCl. Clinical strains were incubated at 37°C, and environmental isolates were incubated at 25°C for 24 h.

DNA isolation. Chromosomal DNA was extracted by the guanidium thiocyanate method of Pitcher et al. (29) and further purified by RNase and proteinase K treatments (only for ribotyping) as described by Sambrook et al. (30).

Ribotyping. Strains were ribotyped as described previously by Aznar et al. (6). In that work, 12 different endonucleases were screened for *V. vulnificus* DNA digestion and two different oligonucleotides were used as probes. The highest degree of resolution was achieved with *KpnI* combined with a probe, 1038, complementary to a highly conserved region of eubacterial 23S rRNA genes. Briefly, 5 µg of chromosomal DNA was digested with the endonuclease *KpnI* as recommended by the manufacturer (GIBCO BRL). The DNA restriction fragments were separated by electrophoresis in 0.8% (wt/vol) agarose gels with TAE (Tris-acetate-EDTA) buffer. DNA was transferred onto a noncharged nylon membrane (Qiabran; Qiagen) under a vacuum (Vacu-Aid System; Hybaid). After transfer, the DNA was hybridized with 18-mer universal probe 1038. The probe was labeled with digoxigenin (DIG Oligonucleotide 3'-End Labeling Kit; Boehringer GmbH, Mannheim, Germany). Hybrid detection was performed by chemiluminescence (DIG Luminescent Detection Kit; Boehringer). The hybridization temperature was 48°C.

Ribotyping analysis. Band patterns displayed on X-Omat 5 films (Kodak) were recorded by an image capture system (Gel Station; TDI, Madrid, Spain) and analyzed by the PC-Windows software package Gel Compare 3.1 (Applied Maths, Kortrijk, Belgium). To obtain an objective comparison and normalization of the bands on different slab gels, a digoxigenin-labeled molecular weight marker was included in each gel at least three times. A mixture of fragments from λ DNA cleaved with *HindIII* (Boehringer) was chosen as the reference pattern because it covers the entire ribopattern area. Digitized images were normalized and combined as described by Vauterin and Vauterin (35). A similarity matrix was created by using the Dice similarity coefficient, S_D (32). For band comparison, a band position tolerance value of 0.8% was allowed to compensate for misalignment of homologous bands due to technical imperfections. The unweighted pair group method using average linkages (UPGMA) (35) was used to cluster the patterns.

AFLP. AFLP fingerprints were determined as described by Vos et al. (37) and Janssen et al. (20). In short, 1 µg of DNA was digested with 10 U each of *HindIII* and *TaqI* (Pharmacia Biotech, Uppsala, Sweden) in a final volume of 30 µl. Following digestion, *HindIII* and *TaqI* adapters were added to final concentrations of 0.04 and 0.4 µM, respectively, and ligated to the restriction fragments by using T4 ligase (Pharmacia Biotech). *HindIII* and *TaqI* adapters were prepared by mixing equimolecular amounts of the partly complementary oligonucleotide sequences 5'-ACGTGGTACGACGTC-3' and 5'-CTCGTAGACTGCGTACC-3' (for *HindIII*) and 5'-GACGATGAGTCGTGAC-3' and 5'-CGGTACAGGAC TCAT-3' (for *TaqI*). DNA was precipitated in the presence of 1.25 M ammonium acetate and 50% (vol/vol) 2-propanol. The DNA pellet was washed once with 70% (vol/vol) ethanol and finally dissolved in 100 µl of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Template DNAs were stored at -20°C.

AFLP reactions employed two specific primers, oligonucleotide 94T02 (5'-C GATGATCCTGACCGAA-3'), corresponding to the *TaqI* ends, and oligonucleotide 93T02 (5'-GAAGTGGTACGACGTTA-3'), corresponding to the *HindIII* ends (selective bases at the 3' ends of the primers are underlined). Primer 93T02 was radioactively end labeled by using [γ -³²P]ATP (Amersham International) and T4 polynucleotide kinase (Pharmacia Biotech) as described previously (37). PCRs were performed in a total volume of 20 µl containing 6.25 ng of labeled primer, 30 ng of unlabeled primer, 5 µl of template DNA, 0.6 U of *Taq* polymerase, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, and each of the deoxynucleoside triphosphates at 0.2 mM. Negative controls (no template) were included in every PCR set. PCR amplifications were performed on a Perkin-Elmer 9600 thermocycler (Perkin-Elmer Corp., Norwalk, Conn.) with the following cycle profile: cycle 1, 60 s at 94°C, 30 s at 65°C, and 60 s at 72°C; cycles 2 to 12, 30 s at 94°C, 30 s at an annealing temperature 0.7°C lower than that used for each previous cycle, starting at 64.3°C, and 60 s at 72°C; cycles

13 to 24, 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C. After completion of the cycling program, an equal volume (20 µl) of 98% (wt/vol) formamide-10 mM EDTA-0.1% (wt/vol) bromophenol blue-0.1% (wt/vol) xylene cyanol was added to the reaction mixtures. Prior to gel loading and electrophoresis, mixtures were heated for 3 min at 95°C and then rapidly cooled on ice to prevent nucleic acid secondary structures from reannealing. PCR products were electrophoretically separated in a denaturing 5% polyacrylamide sequencing gel (obtained as pre-mixed SequaGel solutions from National Diagnostics) and visualized autoradiographically by exposure to Hyperfilm-MP (Amersham International, Buckinghamshire, England) as previously described by Janssen et al. (20). Autoradiographs were scanned with a high-resolution RayVen RSU1 densitometric scanner (X-Ray Scanner Corporation, Torrance, Calif.). Transmission image data were stored as tagged image format files.

AFLP analysis. The fingerprint patterns were analyzed by using the PC-Windows software package Gel Compare, version 3.1, mentioned above. A reference strain was included every six tracks as a standard for objective comparison and normalization of patterns in different gels. Following conversion, normalization, and background subtraction by using mathematical algorithms, levels of similarity between AFLP fingerprints were calculated by using the Pearson product-moment correlation coefficient. A cluster analysis was performed by using the UPGMA algorithm.

RESULTS

Ribotyping. Nineteen ribotypes were distinguished among the 80 strains of *V. vulnificus* tested (Fig. 1). They exhibited stable and reproducible patterns consisting of 7 to 10 bands ranging from 26.8 to 2.0 kb (Fig. 2) which correspond to the rRNA operon contents. A band of 12.3 kb was present in 17 of the 19 ribotypes. The second most common band corresponded to 7.7 kb, followed by bands of 5.9, 10.1, and 5.6 kb (Fig. 2). Cluster I joins 19 of 44 biotype 1 strains at 81% similarity, these strains exhibited quite similar ribotypes named G, H, and I, and all of them were isolated at the same fish farm. Fourteen strains belonged to ribotype I, four belonged to ribotype H, and one belonged to ribotype G. Cluster II is defined at 76% similarity and groups biotype 2 strains which showed three slightly different ribotypes named J, K, and R (Fig. 1) with a biotype 2-specific band of 2.8 kb (Fig. 2). Thirty-three of the 36 strains in this group belonged to ribotype J, 2 strains belonged to ribotype K, and only 1 belonged to ribotype R. Their nearest neighbor is cluster III, which groups, at 69% similarity, ribotypes A, C, L, N, O, and S, corresponding to 16 biotype 1 strains, including the type strain of *V. vulnificus*. The remaining ribotypes showed higher heterogeneity.

AFLP analysis. Figure 3 shows the results of the cluster analysis of the AFLP patterns. It is noteworthy that this technique yielded strain-specific patterns of 80 to 100 bands. Interestingly, all of the biotype 2 strains recovered from diseased eels, which exhibited the main phenotypic trait of biotype 2 (indole negative), grouped at 72% similarity, forming the tightest group (cluster I). Within this group, several characteristic profiles could be related to different outbreaks in the same eel farm and/or to specific phenotypic traits, i.e., atypical biotype 2 strains positive for ornithine decarboxylase (ER1, R1, R2, E103, E105, E106, VIB 522, VIB 525, VIB 526, and VIB 527) (Fig. 3). Apart from them, two atypical biotype 2 strains, positive for indole production (171 and VIB 523) but isolated from diseased eels, were grouped with biotype 1 strains recovered from healthy animals at different geographical sites. Biotype 1 strains isolated from healthy fish at the Spanish eel farm grouped in clusters II and III at 92 and 78% similarity, respectively. The low similarity shown by clinical isolates is noteworthy. No relationship with the geographic origin or source of the isolate was observed.

DISCUSSION

Several approaches have been used to analyze the intraspecific diversity of *V. vulnificus*. Among the recently developed

TABLE 1. Strains used in this study

Strain	Source (yr of isolation)	Country	BT ^a	RT ^b	AFLP cluster
ATCC 27562	Human wound infection	USA ^c	1	A	
C7184	Human blood	USA	1	B	
UMH1	Fatal wound infection	USA	1	C	
Vv1	Fatal wound infection	USA	1	D	
374	Septicemia case	USA	1	E	
TW1	Fish farm tank water	Spain	1	F	
LMG 12095	Eel	Belgium	1	F	
M631	Eel (1990)	Spain	1	G	III
D10	Sea bream (1990)	Spain	1	H	II
M76	Eel (1990)	Spain	1	H	II
E109	Eel (1990)	Spain	1	H	II
E110	Eel (1990)	Spain	1	H	III
M40	Eel (1990)	Spain	1	I	III
M47	Eel (1990)	Spain	1	I	III
M63	Eel (1990)	Spain	1	I	III
M79	Eel (1990)	Spain	1	I	III
M89	Eel (1990)	Spain	1	I	III
M90	Eel (1990)	Spain	1	I	III
M324	Eel (1990)	Spain	1	I	III
M328	Eel (1990)	Spain	1	I	III
M335	Eel (1990)	Spain	1	I	III
M626	Eel (1990)	Spain	1	I	III
M647	Eel (1990)	Spain	1	I	III
E112	Eel (1990)	Spain	1	I	II
E113	Eel (1990)	Spain	1	I	III
E114	Eel (1990)	Spain	1	I	III
ATCC 33149	Diseased eel	Japan	2	J	I
NCIMB 2136	Diseased eel	Japan	2	J	I
NCIMB 2137	Diseased eel	Japan	2	J	I
E4	Diseased eel (1989)	Spain	2	J	I
E12	Diseased eel (1989)	Spain	2	J	I
E22	Diseased eel (1989)	Spain	2	J	I
E24	Diseased eel (1989)	Spain	2	J	I
E32	Diseased eel (1989)	Spain	2	J	I
E37	Diseased eel (1989)	Spain	2	J	I
E39	Diseased eel (1989)	Spain	2	J	I
E40	Diseased eel (1989)	Spain	2	J	I
E52	Diseased eel (1989)	Spain	2	J	I
E56	Diseased eel (1989)	Spain	2	J	I
E58	Diseased eel (1989)	Spain	2	J	I
E64	Diseased eel (1990)	Spain	2	J	I
E77	Diseased eel (1990)	Spain	2	J	I
E80	Diseased eel (1990)	Spain	2	J	I
E86	Diseased eel (1990)	Spain	2	J	I
E92	Diseased eel (1990)	Spain	2	J	I
M206	Diseased eel (1990)	Spain	2	J	I
M338	Diseased eel (1990)	Spain	2	J	I
E103	Diseased eel (1990)	Spain	2	J	I
E105	Diseased eel (1990)	Spain	2	J	I
E106	Diseased eel (1990)	Spain	2	J	I
ER1	Diseased eel (1992)	Spain	2	J	I
R1	Diseased eel (1994)	Spain	2	J	I
R2	Diseased eel (1994)	Spain	2	J	I
VIB 524	Diseased eel	Norway	2	J	I
VIB 525	Diseased eel	Sweden	2	J	I
VIB 526	Diseased eel	Sweden	2	J	I
VIB 527	Diseased eel	Sweden	2	J	I
VIB 523	Diseased eel	Sweden	2	J	I
171	Diseased eel	Belgium	2	J	
NCIMB 2138	Diseased eel	Japan	2	K	I
LMG 13638	Shrimp	Unknown	2	K	
143	Eel	Belgium	1	L	
LMG 12090	Eel	Belgium	1	L	
147	Eel	Belgium	1	L	
149	Eel	Belgium	1	L	
LMG 12092	Eel	Belgium	1	L	
155	Eel	Belgium	1	L	
161	Eel	Belgium	1	L	

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TABLE 1—Continued

Strain	Source (yr of isolation)	Country	BT ^a	RT ^b	AFLP cluster
161	Eel	Belgium	1	L	
169	Eel	Belgium	1	L	
160	Eel	Belgium	1	M	
167	Eel	Belgium	1	N	
248	Eel	Belgium	1	N	
249	Eel	Belgium	1	N	
170	Human blood	Belgium	1	O	
LMG 13637	Shrimp	Thailand	1	P	
VIB 537	Shrimp	Thailand	1	P	
VIB 521	Unknown	Unknown	1	Q	
VIB 522	Human blood	Unknown	2	R	
VIB 535	Eel	Sweden	1	S	
VIB 536	Eel	Sweden	1	S	

^a BT, biotype.^b RT, ribotype.^c USA, United States of America.

genomic fingerprinting techniques, PFGE and ribotyping have been successfully applied to biotype 1 strains, revealing a high level of intraspecific diversity, especially PFGE (11, 33). Aznar et al. (6) used ribotyping and random amplification of polymorphic DNA for a few biotype 1 and 2 strains and found specific profiles for both biotypes, biotype 2 being more homogeneous. In the present study, we have used ribotyping and AFLP in a higher number of strains of both biotypes from different geographic origins to obtain a reliable picture of their genetic variability, as well as to compare the two techniques.

Despite their different degrees of resolution, both methods have confirmed that biotype 2 isolates, irrespectively of their geographic origins, are highly homogeneous; this is explained by the fact that practically all biotype 2 strains have been isolated from the same type of source: diseased eels. In contrast, the biotype 1 isolates recovered from healthy eels showed great genomic diversity, which is in accordance with the results obtained by Buchrieser et al. when analyzing individual oysters (11). Although three different ribotypes were found in our biotype 2 isolates, 96% shared a profile (J) that was shown by all of the strains recovered from the same eel farm in successive outbreaks. This profile could therefore be used to trace them for epidemiological studies. Biotype 1 isolates displayed up to 16 ribopatterns, a finding which stresses the heterogeneity already observed for this biotype by phenotypic approaches (2, 7). Such diversity has been also observed by other researchers who have recently used *Hind*III and probed with cDNA of

16 and 23S rRNAs from *Escherichia coli* for discrimination among biotype 1 isolates from clinical and environmental origins (33). In the present study, and despite the high diversity found for biotype 1 isolates, ribotypes I, G, and H, which cluster together (cluster I), correspond to strains isolated from the same farm. In addition, they were close to the ribotypes exhibited by the eel-pathogenic strains of cluster II. In all, four

Ribotypes

FIG. 1. Ribotypes within *V. vulnificus*. Clustering of *Kpn*I ribotypes is shown. Degrees of similarity are indicated at the top. The capital letters correspond to the ribotype profiles listed in Table 1. Clusters I, II, and III are marked.

FIG. 2. Schematic representation of the *Kpn*I ribotypes obtained for the 80 strains of *V. vulnificus* analyzed.

FIG. 3. AFLP patterns of *V. vulnificus*. The dendrogram was derived by UPGMA cluster analysis of the AFLP patterns of 80 *V. vulnificus* strains. The tracks show the processed band patterns after conversion, normalization, and subtraction of the background. Levels of linkage are expressed as the Pearson product-moment similarity coefficient. Clusters I, II, and III are indicated. Biotype 2 strains are underlined.

ribotypes, three for biotype 1 and one for biotype 2, represent the Spanish eel farm isolates, and their common source of isolation might explain their similarity. No other relationship between biotype 1 strains and geographic origins or sources of isolation could be inferred from the ribopattern analysis.

With regard to AFLP fingerprinting, the strain-specific pattern allowed the differentiation of subgroups within the eel-pathogenic isolates sharing the same ribopattern, i.e., J. Within this group, strains corresponding to the successive outbreaks that occurred in the fish farm between 1989 and 1990 showed similar profiles, confirming the results obtained by ribotyping. A slightly different pattern was displayed by some biotype 2 strains that showed an atypical phenotype (presence of ornithine decarboxylase). They corresponded to the Spanish isolates recovered from the last outbreaks that occurred at the end of 1990 and the sporadic ones recorded during the last outbreaks, as well as three Swedish isolates. Since these strains were all grouped within ribotype J, it can be concluded that better discrimination among strains from the same source (a Spanish eel farm) was obtained by AFLP. The fact that some AFLP clusters included more than one ribotype means that no relationship between AFLP subclusters and ribotypes could be established. The same conclusion about ribotyping and PFGE, another powerful technique for molecular fingerprinting, has recently been established by Tamplin et al. (33) for *V. vulnificus*. Like PFGE, AFLP revealed a high level of intraspecific diversity, especially with clinical and environmental isolates.

In view of the results of the present study, both ribotyping and AFLP constitute good genetic markers for intraspecific differentiation of *V. vulnificus* with different degrees of resolution. Although no marker fulfills all of the requirements for epidemiological-ecological comparison of bacterial strains, ribotyping offers a clear advantage over other molecular methods because it is based on the rRNA sequences highly conserved among eubacteria. In addition, the presence of several copies of the rRNA operon on the chromosome of *V. vulnificus* (6) allows the display of a reasonable number of bands (from 7 to 10). In our study, we were able to clearly separate eel-pathogenic strains (biotype 2) from clinical and environmental isolates (biotype 1) with this technique.

With AFLP, we were even able to differentiate single isolates within biotypes, maintaining the already observed separation between the two biotypes but allowing finer discrimination. AFLP combines the reliability of RFLP with the power of PCR; it detects genomic restriction fragments and in that aspect resembles the RFLP procedure, but instead of Southern hybridization for detection of restriction fragments, it uses amplification by PCR. In this case, the differentiation level depends on the combination of restriction enzymes and amplification primers. We have used primers with one selective nucleotide, but it is possible to add up to three bases and maintain selective amplification. These changes would increase or decrease the number of bands in the patterns, tuning the discrimination level (20, 37). In any case, the analysis of the high number of bands yielded by this method makes it necessary to use one of the available software packages, such as Gel Compare 3.1. By this procedure, we were able to obtain a strain-specific pattern and even to differentiate successive outbreaks that occurred at a Spanish eel farm over several years of study and were indistinguishable by ribotyping. AFLP there-

fore constitutes one of the most discriminative tools for the monitoring of individual strains for epidemiological and ecological studies.

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